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14. ABSTRACT

Angiogenin is an angiogenic ribonuclease that is upregulated in prostate cancer. The objective of this project is to explore the role angiogenin plays in the development of androgen-independent disease. The hypothesis to be tested is that angiogenin plays an essential role in rRNA transcription in prostate cancer cells and that constitutive nuclear translocation of angiogenin is a driving force for transition to androgen independence. Toward this goal, we have carried out experiments to show that overexpression of angiogenin in androgen-dependent LNCaP prostate cancer cells enable them to grow in the absence of androgen, and that knocking-down angiogenin expression in androgen-independent PC-3 prostate cancer cells inhibits cell proliferation in vitro and in vivo, accompanied with a decrease in both cancer cell proliferation and angiogenesis. These results are consistent with our hypothesis and indicate that angiogenin is a novel therapeutic target for prostate cancer.

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Angiogenin, androgen-independent prostate cancer, therapeutic target

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Introduction

The goal of this study is to understand the molecular mechanism of angiogenin-mediated growth of androgen- and androgen receptor-independent growth of prostate cancer. This goal will be achieved by two specific aims. Specific aim 1 is to demonstrate the role of angiogenin in prostate cancer by characterizing the growth of angiogenin over-expressing LNCaP cells in castrated athymic mice and angiogenin under-expressing PC-3 cells in non-castrated mice. Specific aim 2 is to elucidate the mechanism by which angiogenin stimulates rRNA transcription. These two specific aims will be accomplished in 6 tasks. The first year covers task 1 and part of task 2 as described in the "Statement of Work" of the original proposal. We have accomplished task 2 and have made substantial progress in task 1. The work carried out in this period has generated a paper in the *Proceedings of the National Academy of Sciences of the United States of America.*

Body

Task 1: Task 1 is to characterize the growth of angiogenin over-expressing LNCaP cell in castrated athymic mice. We have transfected angiogenin expression vector into LNCaP cells, selected stable transfectants, and shown that angiogenin over-expressing LNCaP cells are able to grow in vitro in androgen-independent manner.

1. Exogenous angiogenin stimulates proliferation of LNCaP cells but not that of PC-3, PC-3M, and DU145 cells.

Nucleolar angiogenin in both endothelial cells (*1*) and HeLa cells (*2*) has been shown to stimulate rRNA transcription, a rate-limiting step in ribosome biogenesis and cell proliferation. The hypothesis to be tested in this study is that nuclear translocation of angiogenin is associated with androgen-independent growth of prostate cancer cells, especially with androgen receptor (AR)-independent growth such as that of PC-3 cells. In the presence of a functional AR, rRNA transcription is mediated by androgen activated AR, as has been demonstrated in animals (*3, 4*) and in LNCaP cells (*5*). When prostate cancer progresses to the AR-independent state, rRNA transcription has to be continually increased and may be mediated by constitutive nuclear translocation of angiogenin. To test this hypothesis, we first examined the effect of exogenous angiogenin on the proliferation of androgen-dependent cells (LNCaP) and androgen-independent cells (PC-3, PC-3M, and DU145) in the absence and presence of androgen.

Fig. 1 shows that LNCaP proliferation was stimulated by exogenous angiogenin when cultured in steroid-depleted medium. The proliferation activity of angiogenin was not quite as much as that of dihydrotestosterone (DHT), probably due to a wide spectrum effect of androgen on cell proliferation which cannot be completely replaced by angiogenin stimulation. However, the fact that angiogenin has approximately 60% of the activity of DHT in stimulating LNCaP proliferation indicates that angiogenin can at least partially compensate for the loss of androgen-deprivation. Moreover, angiogenin and DHT were not additive, suggesting that angiogenin may share one of the mechanisms by which androgen modulates cell proliferation. On the other hand, exogenous angiogenin has no effect on proliferation of PC-3 (Fig. 1B), PC-3M (Fig. 1C), and DU145 (Fig. 1D) cells, perhaps because the nuclei of these cells already have adequate amount of endogenous angiogenin. This conclusion is also supported by the data in the next section where we show that angiogenin over-expression promotes LNCaP cell proliferation (Fig. 2), whereas knocking-down angiogenin expression inhibits PC-3 cell proliferation (Fig. 3).

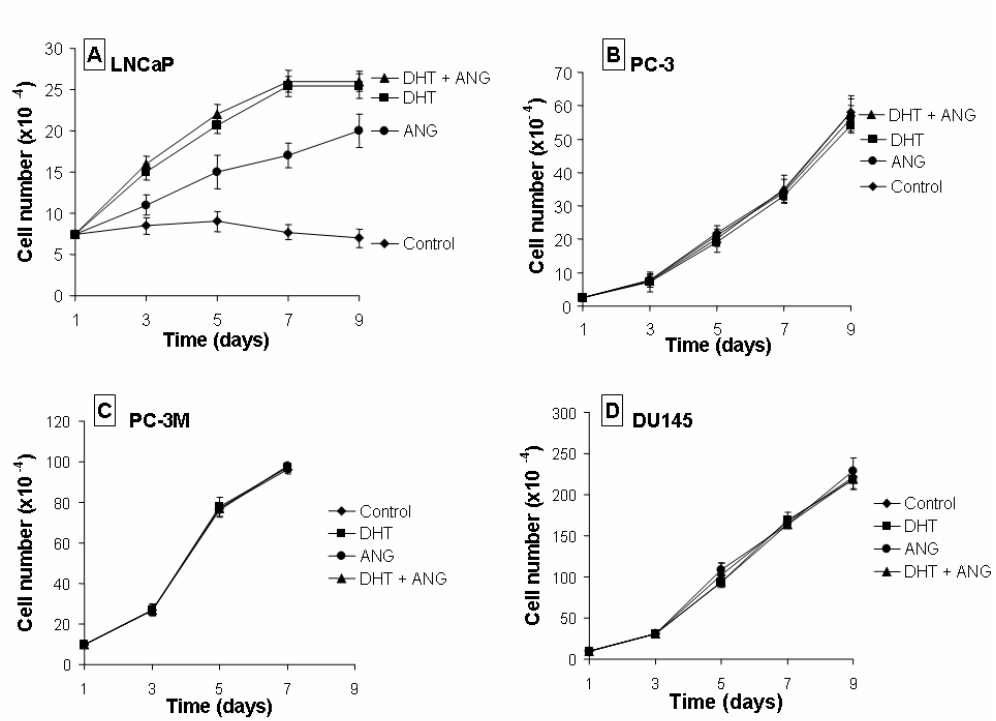

Fig. 1. Effect of exogenous angiogenin on prostate cancer cell proliferation. LNCaP (***A***), PC-3 (***B***), PC-3M (***C***), and DU145 (***D***) cells were cultured in RPMI 1640 (***A***) and DMEM (***B-D***), respectively, + 10% FBS for 24 h. They were washed three times with serum-free medium and cultured in 10% charcoal/ dextran-stripped FBS. DHT (10 nM), angiogenin (1 μg/ml), or a mixture of the two were added and the cells were cultured for the time periods indicated. Cell numbers were determined with a Coulter counter. Data shown are from a representative experiment of three independent repeats.

2. Over-expression of angiogenin in LNCaP cells promotes androgen-independent proliferation

The angiogenin expression vector pCI-ANG that carries human angiogenin cDNA controlled by the CMV promoter and the control vector pCI-Neo were transfected into LNCaP cells, together with a GFP encoding plasmid pEGFP-C1, with Lipofectamine. Stable transfectants were selected by G418 and the GFP expressing cells were sorted by FACS. Chromosomal integration of the transfected gene was confirmed by genomic DNA PCR with a forward primer from the T7 sequence of the pCI vector and a reverse primer from the inserted *angiogenin* sequence. The angiogenin expression levels in pooled populations of vector and angiogenin transfectants were 0.50 and 3.03 ng/10^6 cells/day, respectively, as determined by ELISA analysis (Fig. 2). The vector control and angiogenin transfectants (pooled population) were seeded at 1x10^4 cells per 35-mm dish and cultured in RPMI 1640 + 10% FBS for 24h. The cells were washed with serum-free medium 3 times and switched to steroid-free medium (RPMI +10% charcoal/dextran-stripped FBS). As shown in Fig. 2, angiogenin over-expression stimulated LNCaP proliferation ~3-fold. To determine whether angiogenin over-expression will promote androgen-independent proliferation in vivo, the pCI-Ang transfectants and pCI control transfectants will be inoculated into castrated athymic BALB/c-nu mice (10 weeks old), and their growth rates will be determined.

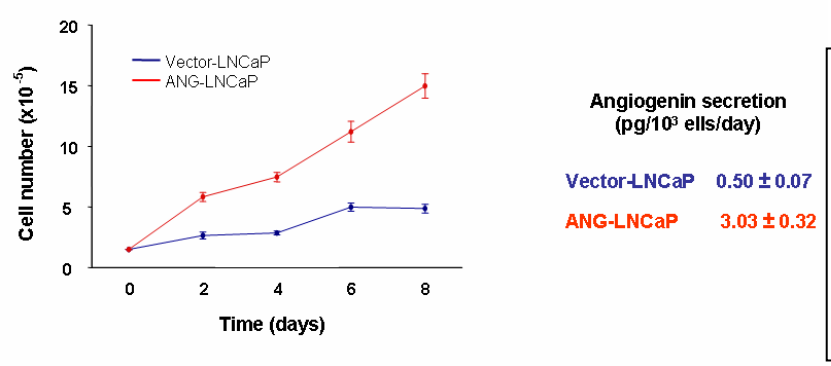

Fig. 2. Angiogenin over-expression promotes LNCaP proliferation. Vector control (brue) and angiogenin (red) transfectants were cultured in the absence of androgen (RPMI + 10% charcoal /dextran-stripped FBS). Cell numbers were determined with a Coulter counter.

Task 2: Task 2 is to characterize the growth of angiogenin under-expressing PC-3 cells in athymic mice. We have transfected an angiogenin RNAi plasmid into PC-3 cells and have characterized the in vitro and in vivo growth of angiogenin under-expressing PC-3 cells. The results obtained in this task have demonstrated an essential role of angiogenin in PC-3 cell growth both in vitro and in vivo.

Down-regulation of angiogenin in PC-3 cells inhibits cell proliferation and tumorigenesis.

To examine the role of angiogenin in androgen-independent prostate cancer cell proliferation, we used a plasmid-mediated RNAi method to knockdown angiogenin expression in PC-3 cells and measured the resultant changes in rRNA transcription and in cell proliferation. pANG-RNAi that specifically targets human angiogenin mRNA at nt 381-401 has been used successfully in our previous work to knockdown angiogenin expression in HeLa cells (*2*). As shown in Fig. 3A, stable transfection of pANG-RNAi in PC-3 cells decreased angiogenin expression from 0.65 (vector control pBS/U6 transfectants) to 0.15 ng/10^6 cells/day, representing a 77% reduction. There was a concomitant decrease in the steady-state level of 45S rRNA (Fig. 3B, middle lane) as determined by Northern blotting analysis. Exogenous angiogenin (1 μg/ml) was able to restore the 45S rRNA level to that of the vector control (Fig. 3B, right lane), indicating that the inhibitory effect was mediated by down-regulating angiogenin rather than by a nonspecific effect of plasmid transfection.

A metabolic labeling experiment was carried out to determine the effect of angiogenin down-regulation on the *de novo* synthesis of the rRNA. Vector and RNAi transfectants were pulse-labeled with ^{32}P-orthophosphate for 30 min. The newly synthesized 45S rRNA was detected by autoradiography (Fig. 3C, top), and the level of 28S rRNA was determined by EB staining (Fig. 3C, bottom). pANG-RNAi transfectants have a reduced rate of *de novo* synthesis of 45S rRNA as compared to that of pBS/U6 vector control transfectants. Exogenous angiogenin was able to reverse the inhibition (Fig. 3C, right lane). EB staining of 28S rRNA showed that equal amounts of total RNA were applied in each lane. Because of the large cytoplasmic pool of stable ribosomes, the cellular levels of 28S and 18S rRNA were relatively stable (*6*). Thus, 28S rRNA still serves as the best loading control in these experiments. However, it should be noted that the RNAi transfectants do have a reduced steady-state level of 45S rRNA, which results in a decrease in the cellular level of 28S and 18S rRNA. Therefore, using 28S rRNA as a loading control could have underestimated the difference in newly synthesized 45S rRNA per cell between RNAi and vector transfection. In any event, the data presented in Fig. 3B and 3C clearly demonstrate that down-regulating angiogenin expression in PC-3 cells inhibits 45S rRNA transcription.

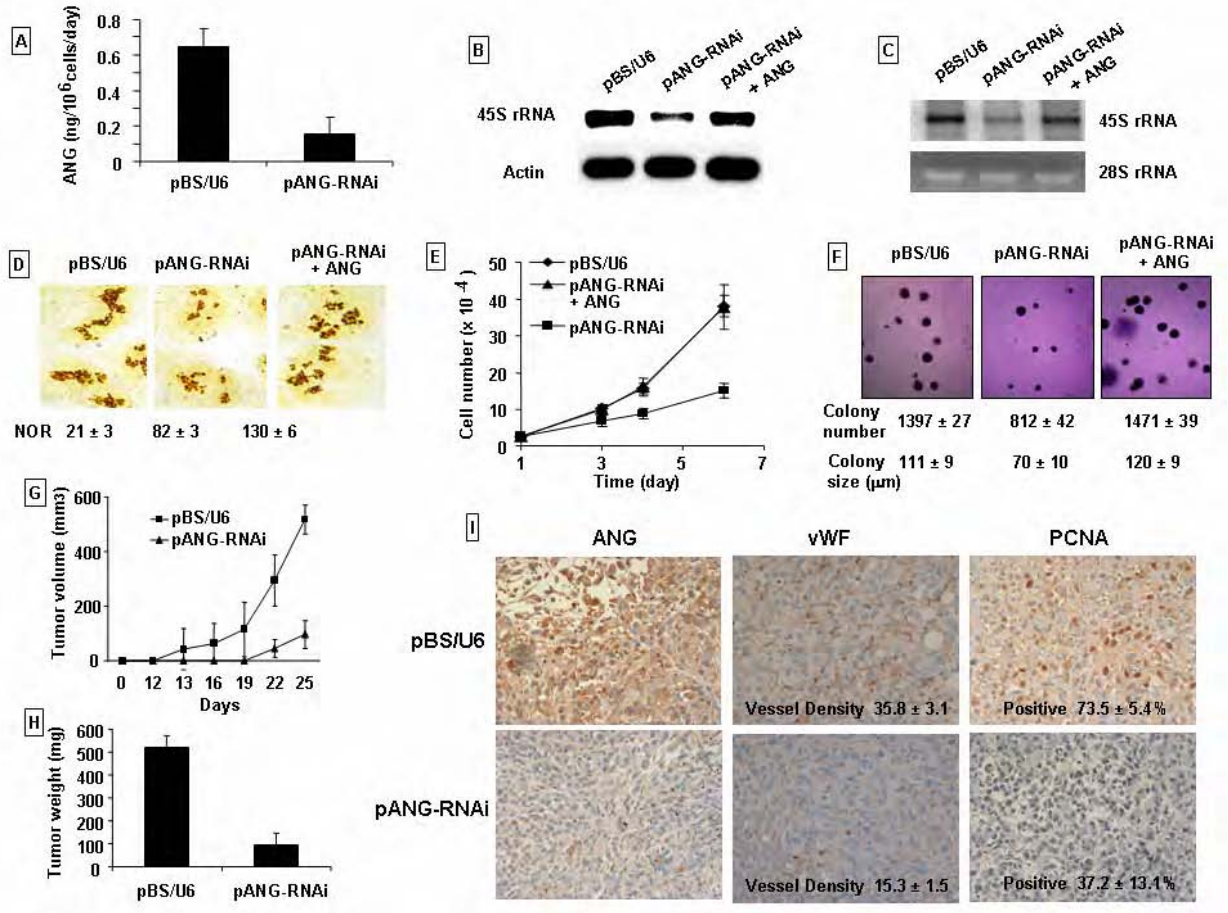

Fig. 3. Down-regulation of angiogenin inhibits PC-3 cell growth. PC-3 cells were transfected with angiogenin RNAi plasmid pANG-RNAi or the vector control pBS/U6. Stable transfectants were selected as described for HeLa cells (Tsuji et al., 2005). ***A***: Secreted angiogenin level determined by ELISA. ***B***: The steady-state level of 45S rRNA determined by Northern blotting with actin mRNA as the loading control. ***C***: The *de novo* synthesis of 45S rRNA after 30 min pulse-labeling with 25 μCi/ml ^{32}P-orthophosphate. Radiolabeled RNA was visualized by autoradiography. 28S rRNA was stained with EB. ***D***: Silver staining of nuclear organizer regions (NOR). The numbers shown beneath the pictures are the average number of NOR per nucleus determined from 30 random cells. ***E***: Cell proliferation as determined with a Coulter counter. When present, exogenous angiogenin was 1 μg/ml. ***F***: Soft agar assay in which cells were seeded at a density of 4 x 10^3 cells per 35-mm dish, and cultured in 0.35% soft agar in DMEM + 10% FBS at 37 ^{o}C for 7 days. When present, angiogenin was added to both the soft agar and the medium at 0.1 μg/ml. The colonies were stained with 0.05% crystal violet. Colony numbers in the entire dish were counted. The average colony size was determined by measuring the diameters of colonies in 10 microscope fields with a micro-caliper. ***G and H:*** tumor growth in nude mice. 1x10^6 cells per mouse were inoculated s.c. (6 mice per group). Mice were checked daily for tumor appearance by palpation and tumor volume was measured every three days (***G***). Tumors were removed day 31 and weighed (***H***). ***I***: Immunohistochemistry staining of angiogenin, neovessels, and PCNA. Thin sections (4 μm) from formalin-fixed, paraffin-embedded tumor tissues were stained with anti-ANG, anti-vWF and anti-PCNA antibodies and visualized with Dako's Envision system. vWF-positive vessels in each tumor were counted in five most vascularized areas at 200 x and the number averaged. Vessel density (vessels/field) is shown as mean ± SD for each group. PCNA positive and total numbers of cells were counted in 5 random areas at 200 x. Pictures shown were from a representative animal of each group.

Nuclear organizer regions (NOR) are loops of rDNA that are being actively transcribed for rRNA. These regions are thus involved in the synthesis of ribosomes and are of central importance in the regulation of protein synthesis (*7*). The size and number of NOR reflect the capacity of the cell to transcribe rRNA (*8*). NOR are associated with a group of so-called argyrophilic proteins and can be visualized by silver staining. Fig. 3D shows that pANG-RNAi transfectants have significantly reduced ($P<0.0001$) NOR numbers, which can be restored by adding exogenous angiogenin. These results demonstrate that down-regulating angiogenin expression in PC-3 cells specifically inhibits ribosome biogenesis.

Because of the central importance of rRNA transcription in cell growth, decreased rRNA transcription should attenuate cell proliferation. This is confirmed by cell proliferation assays (Fig. 3E). Cell number counting showed that pANG-RNAi transfectants have a reduced proliferation rate compared to that of the pBS/U6 vector transfectants. Again, exogenous angiogenin was able to restore cell proliferation to the level of vector control.

The anchorage-independent growth of pANG-RNAi transfectants was analyzed by a colony formation assay in soft agar (Fig. 3F). RNAi transfection decreased both colony number ($P<0.0001$) and size ($P<0.001$) significantly. Complete recovery was obtained when exogenous angiogenin was added (Fig. 3F, right panel).

The effect of knocking-down angiogenin on *in vivo* growth of PC-3 cells was examined in athymic mice. All the animals in both groups developed tumors. However, the growth rate in the knockdown group was substantially lower than that in the vector control group (Fig. 3G). The average tumor weight after 31 days in pANG-RNAi mice was 96 ± 51 mg, representing a 82% reduction from the pBS/U6 mice (518 ± 52 mg) (Fig. 3H). Immunohistochemistry staining with anti-angiogenin mAb 26-2F confirmed that the PC-3 tumors derived from pANG-RNAi transfectants have lower angiogenin level (Fig. 3I, left panel). vWF and PCNA staining show that they also have decreased vessel density (Fig. 3I, middle panel) and less proliferating cells (Fig. 3I, right panel). These results demonstrate that down-regulating angiogenin expression decreased tumorigenecity of PC-3 cells by an inhibition of both tumor angiogenesis and cancer cell proliferation.

Together, these results demonstrate that endogenous angiogenin in PC-3 cells plays an important role in rRNA transcription, ribosome biogenesis, cell proliferation and tumorigenesis.

Key Research Accomplishment

- Confirmed that angiogenin stimulates proliferation androgen-dependent prostate cancer cells (LNCaP) but not androgen-independent prostate cancer cells including PC-3, PC-3M, and DU145.
- Generated angiogenin transfectant LNCaP cells that express 6 times (3.03 ng/10^6 cells/day) more angiogenin than does the vector control transfectants (0.5 ng/10^6 cells/day).
- Demonstrated that angiogenin over-expression LNCaP cells are able to grow in the absence of androgen and other steroid hormones.
- Confirmed that knocking-down angiogenin in PC-3 cells inhibits rRNA synthesis, ribosome biogenesis, anchorage-dependent and -independent in vitro growth.
- Demonstrated that angiogenin knockdown PC-3 cells have a reduced growth rate in vivo (xenograft model in athymic mice) accompanied by a decrease in both angiogenesis and cancer cell proliferation.

Reportable outcomes

A paper entitled "A therapeutic target for prostate cancer based on angiogenin-stimulated angiogenesis and cancer cell proliferation" has been published in *Proceedings of the National Academy of Sciences of the United States of America*, **103** (39) 14519-14524, 2006.

Conclusion

The completed work in the period from Oct. 15/2005 to Oct 14, 2006 has demonstrated that angiogenin plays an important role in prostate cancer. The results indicated that the function of angiogenin in the nuclei of prostate cancer cells is to stimulate rRNA transcription, a primary target of the androgen-androgen receptor signal axis. Thus, upregulation of angiogenin and constitutive nuclear translocation of angiogenin in prostate cancer cells would result in a constant supply of rRNA, which may be one of the causative factors for the development of androgen independency. The results indicated that angiogenin would be a therapeutic target for androgen-independent prostate cancer.

The research is on track. No changes or modifications are foreseen for the next 12 months.

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Appendices

Reprint

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A therapeutic target for prostate cancer based on angiogenin-stimulated angiogenesis and cancer cell proliferation

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Human angiogenin is progressively up-regulated in the prostate epithelial cells during the development of prostate cancer from prostate intraepithelial neoplasia (PIN) to invasive adenocarcinoma. Mouse angiogenin is the most up-regulated gene in AKT-induced PIN in prostate-restricted AKT transgenic mice. These results prompted us to study the role that angiogenin plays in prostate cancer. Here, we report that, in addition to its well established role in mediating angiogenesis, angiogenin also directly stimulates prostate cancer cell proliferation. Angiogenin undergoes nuclear translocation in PC-3 human prostate cancer cells grown both *in vitro* and in mice. Thus, knocking down angiogenin expression in PC-3 human prostate adenocarcinoma cells inhibits ribosomal RNA transcription, *in vitro* cell proliferation, colony formation in soft agar, and xenograft growth in athymic mice. Blockade of nuclear translocation of angiogenin by the aminoglycoside antibiotic neomycin inhibited PC-3 cell tumor growth in athymic mice and was accompanied by a decrease in both cancer cell proliferation and angiogenesis. These results suggest that angiogenin has a dual effect, angiogenesis and cancer cell proliferation, in prostate cancer and may serve as a molecular target for drug development. Blocking nuclear translocation of angiogenin could have a combined benefit of antiangiogenesis and chemotherapy in treating prostate cancer.

tumor therapy | nuclear translocation | ribosome biogenesis

Angiogenin is a 14-kDa angiogenic ribonuclease originally isolated from HT-29 colon adenocarcinoma cells (1). Its expression is up-regulated in various types of human cancers, including breast (2), cervical (3), colon (4), colorectal (5), endometrial (6), gastric (7), liver (8), kidney (9), ovarian (10), pancreatic (11), prostate (12), and urothelial (13) cancers, as well as astrocytoma (14), leukemia (acute myeloid leukemia and myelodysplastic syndrome) (15, 16), lymphoma (non-Hodgkin's) (17), melanoma (18), osteosarcoma (19), and Wilms' tumor (20). Among them, prostate cancer, in which angiogenin expression is positively correlated with disease progression (12), is of particular interest. Majumder *et al.* (21) reported that the angiogenin protein content in the serum of patients with hormone refractory prostate cancer (40 patients), in newly diagnosed prostate cancer patients (39 patients), and in control patients with no evidence of prostate cancer (37 patients) was 436 ± 24, 392 ± 17, and 328 ± 20 ng/ml, respectively. There is a statistically significant difference in serum angiogenin levels between the controls and untreated, hormone-naïve prostate cancer patients ($P < 0.01$) and between controls and hormone refractory prostate cancer patients ($P < 0.001$). There is also a trend toward higher levels of angiogenin in hormone refractory patients compared with newly diagnosed patients.

It is known that circulating angiogenin in normal plasma is mainly produced by the liver (22) and is at a concentration of 250–350 ng/ml (11, 13). Therefore, if the elevated serum angiogenin level in prostate cancer patients results from an up-regulation of prostatic expression of angiogenin, it reflects a dramatic increase in angiogenin expression in the prostate. Katona *et al.* (12) analyzed a large cohort of 107 radical prostatectomy specimens by immunohistochemistry (IHC) and found that angiogenin expression increases progressively as prostatic epithelial cells evolve from a benign phenotype to an invasive phenotype. This finding was in agreement with an earlier report in which Olson *et al.* (23) showed that angiogenin protein is barely detected in normal prostate tissue (7 patients) and is dramatically increased in prostate adenocarcinoma (10 patients).

The mouse has four angiogenin isoforms that show 76%, 39%, 62%, and 46% identity to human angiogenin (24). Mouse angiogenin-3 is prostate-specific (25) and is the most up-regulated gene detected in prostate epithelial cells in the AKT-induced prostate intraepithelial neoplasia (PIN) lesions observed in murine prostate-restricted Akt kinase transgenic (MPAKT) mice (21). Gene microarray analysis showed that mouse angiogenin-3 had the highest signal-to-noise score and was induced 32-fold in MPAKT mice (21). Mouse angiogenin-1 and -2 were also overexpressed with a 3- and 10-fold induction, respectively. Moreover, mTOR (mammalian target of rapamycin) inhibition reversed Akt-induced PIN in MPAKT mice and partially restored angiogenin-3 expression (26). These results suggest that angiogenin is involved in prostate cancer pathogenesis. In the present study, we examined the effect that knocking down angiogenin expression has on the growth of PC-3 cells both *in vitro* and *in vivo*. We have also examined the antitumor activities of neomycin, which blocks nuclear translocation of angiogenin in both endothelial and cancer cells. Our results suggest that angiogenin plays a role in both tumor angiogenesis and cancer cell proliferation during prostate cancer development.

Results

Enhanced Expression and Nuclear Translocation of Angiogenin in Prostate Cancer. We have examined angiogenin expression levels in human prostate tissues from 23 prostate adenocarcinoma, 20 benign prostate hyperplasia (BPH), and 10 control patients. Fig. 1 shows representative images of IHC staining with the antiangiogenin mAb 26-2F. In normal prostate tissue, angiogenin is detectable in the stroma between the secretory glands (Fig. 1*A*). Angiogenin expression is significantly higher in BPH tissue (Fig. 1*B*) and even higher in prostate cancer tissue (Fig. 1*C*). No angiogenin was detected in the nucleus of the glandular epithelial cells in all 10 normal prostate tissue specimens. But strong nuclear staining was detected in the glandular epithelial cells in BPH

Author contributions: G.-f.H. designed research; N.Y., L.W., K.K., T.T., and G.-f.H. performed research; N.Y. and G.-f.H. analyzed data; and G.-f.H. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: IHC, immunohistochemistry; BPH, benign prostate hyperplasia; ECM, extracellular matrix; PCNA, proliferating cell nuclear antigen; VWF, von Willebrand factor.

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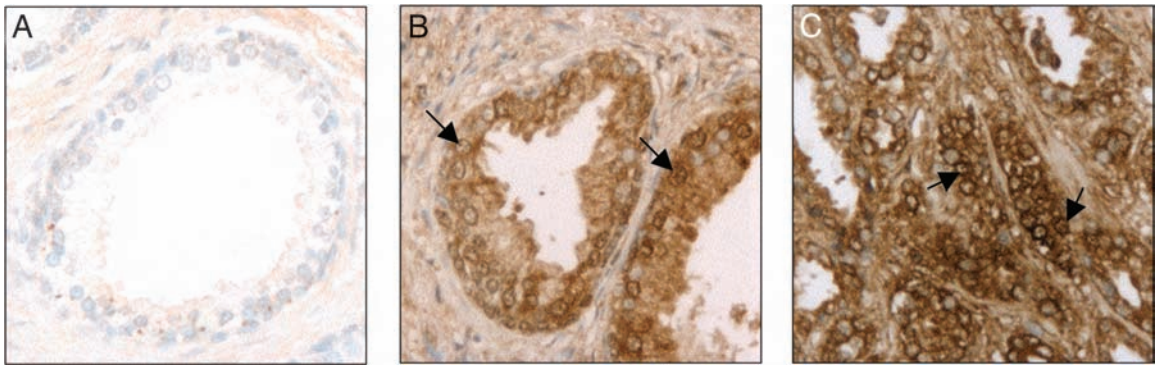

Fig. 1. IHC staining of human angiogenin in prostate tissue samples. Prostate tissue samples from normal (*A*), BPH (*B*), and cancer (*C*) patients were stained with mAb 26-2F (30 μg/ml) and visualized with Dako's Envision kit. (*A*) Weak staining was observed in the stroma in the normal prostate tissue. (*B* and *C*) Enhanced staining was observed in BPH (*B*) and prostate cancer (*C*) tissues, with strong cytoplasmic and nuclear staining. (Magnification: ×400.)

samples (Fig. 1*B*) and in the invasive cancer cells of prostate cancer samples (Fig. 1*C*).

Although angiogenin expression is elevated in many types of human cancer (2–7, 9–20), nuclear angiogenin has so far only been reported in breast cancer tissue samples (27). The strong nuclear angiogenin staining in prostate cancer samples (Fig. 1*C*) prompted us to survey nuclear angiogenin by IHC with a tissue microarray slide containing 35 different human cancer samples. Table 1 lists the categorical results of angiogenin staining in the extracellular matrix (ECM), cytoplasm, and nucleus. Glioma, peripheral nerve sheath tumor, prostate cancer, and cervical cancer tissues have the strongest nuclear staining of angiogenin.

To determine whether nuclear angiogenin observed in these cancer cells is associated with malignant transformation, we surveyed the distribution of angiogenin in a tissue microarray slide containing 30 normal human tissues. Table 2 shows that nuclear angiogenin was detectable in the normal cerebral cortex, peripheral nerve, pancreas, testis, tonsil, and other tissues. No appreciable nuclear angiogenin was detected in normal prostate and cervix samples, although angiogenin was detected in the ECM. Therefore, the prostate and cervix are the two histological sites where nuclear translocation of angiogenin is most significantly increased in tumorigenesis. Consistently, we have shown previously that knocking down angiogenin expression in HeLa cervical cancer cells inhibits cell proliferation and tumor growth in mice (28).

Down-Regulating Angiogenin Expression in PC-3 Cells Inhibits rRNA Transcription and Cell Proliferation. To examine the role of angiogenin in prostate cancer cell proliferation, we knocked down angiogenin expression in PC-3 cells by means of plasmid-mediated RNAi and measured the resultant changes in cell proliferation and tumorigenesis. pANG-RNAi targets human angiogenin mRNA at nucleotides 381–401 and has been used successfully in our previous work to knock down angiogenin expression in HeLa cells (28). As shown in Fig. 2*A*, stable transfection of pANG-RNAi in PC-3 cells decreased angiogenin expression from 0.65 (vector control pBS/U6 transfectants) to 0.15 ng per 10^6 cells per day, representing a 77% reduction.

We have observed previously that the function of nuclear angiogenin is related to rRNA transcription (29), a rate-limiting step in ribosome biogenesis. Knocking down angiogenin expression in endothelial cells (30) and in HeLa cells (28) reduces rRNA transcription, thereby inhibiting cell proliferation. Fig. 2*B* shows that down-regulation of angiogenin expression in PC-3 cells decreases the steady-state level of 47S rRNA (Fig. 2*B*, center lane) as determined by Northern blotting analysis. Exogenous angiogenin (1 μg/ml) was able to restore the 47S rRNA level to that of the vector control (Fig. 2*B*, right lane), indicating that the inhibitory effect was mediated by down-regulating angiogenin rather than by a nonspecific effect of plasmid transfection.

Because of the central importance of rRNA transcription in cell growth, decreased rRNA transcription should attenuate cell proliferation; we confirmed this in a cell proliferation assay (Fig. 2*C*).

Table 1. IHC staining of angiogenin in human cancer tissues

Cancer type	ECM	Cytosol	Nucleus
Glioma	+	+++	++++
Peripheral nerve sheath	+	+++	++++
Prostate	+++	+++	++++
Cervical	+++	+++	++++
Breast	+++	+++	+++
Colon	+++	+++	+++
Seminoma	+++	+++	+++
Renal cell	+++	+++	++
Skin squamous cell	+	+++	++
Rhabdomyosarcoma	+	++	++
Mesothelioma	−	++	++
Ewing's sarcoma	++	+	++
Endometrial	+	+	++
Ovarian yolk sac	++	−	++
Osteosarcoma	+++	+	++
Lung squamous	−	−	++
Thyroid	−	+++	+
Lung adenocarcinoma	++	++	+
Esophagus	+	++	+
Gastric	+++	+	+
Ovarian	+	+	+
Skin basal cell	+	+	+
Synovial sarcoma	−	+	+
Medulloblastoma	−	−	+
Liver	++	+	−
Gastrointestinal stroma	+	+	−
Leiomyosarcoma	−	+	−
Fibrosarcoma	−	+	−
Melanoma	−	+	−
Bladder	++	+	−
Neuroblastoma	++	−	−
Liposarcoma	−	−	−
Hodgkin's disease	−	−	−
B cell lymphoma	−	−	−
T cell lymphoma	−	−	−

The tumor types are listed according to the relative abundance of nuclear angiogenin. +, low; ++, moderate; +++, high; ++++, very high; −, not detectable.

Table 2. IHC staining of angiogenin in normal human tissues

Tissue type	ECM	Cytosol	Nucleus
Tonsil	++++	++	++
Liver	++++	−	−
Breast	++++	−	−
Testis	+++	+	++
Kidney	+++	++	++
Peripheral nerve	+++	+	+++
Esophagus	+++	−	−
Intestine	+++	−	−
Colon	+++	−	−
Prostate	+++	−	−
Cervix	+++	−	−
Ovary	+++	−	−
Thyroid	+++	−	−
Skin	++	+	++
Lung	++	+	−
Heart muscle	++	++	−
Stomach	++	+	+
Cerebellum	++	−	+
Skeleton muscle	++	−	−
Parathyroid	++	−	−
Cerebral cortex	+	+	+++
Pancreas	+	+	+++
Bone marrow	+	+	++
Uterus	+	+	−
Adrenal	+	−	+
Pituitary	+	−	−
Spleen	+	−	−
Omentum	+	−	−
Salivary	−	+++	+
Thymus	−	−	−

The tissues types are listed according to the relative abundance of angiogenin in the ECM because nuclear angiogenin was not significant in most of the samples. +, low; ++, moderate; +++, high; ++++, very high; −, not detectable.

Cell number counting showed that pANG-RNAi transfectants have a reduced proliferation rate compared with that of the pBS/U6 vector transfectants. Again, exogenous angiogenin was able to restore cell proliferation to the level measured for the vector control.

Knocking Down Angiogenin Expression Decreases Tumorigenicity of PC-3 Cells. The anchorage-independent growth of pANG-RNAi transfectants was analyzed by a colony formation assay in soft agar (Fig. 3*A*). Angiogenin RNAi transfection decreased colony number by 42%, from 1,397 ± 27 to 812 ± 42 ($P < 0.0001$). The colony size was also decreased from an average diameter of 111 ± 9 to 70 ± 10 μm ($P < 0.001$). A complete recovery in both the colony number and size was obtained when exogenous angiogenin (0.1 μg/ml) was added (Fig. 3*A Right*), indicating a specific role for angiogenin in anchorage-independent growth of PC-3 cells.

The effect of knocking down angiogenin on *in vivo* growth of PC-3 cells was examined in a xenograft model in *nu/nu* mice. No palpable tumors were detected after 19 days in animals inoculated with angiogenin RNAi transfectants, whereas all of the animals inoculated with the vector control transfectants had tumors. Eventually, all of the animals in both groups developed tumors. However, the growth rate in the knockdown group was substantially lower than that in the control group (Fig. 3*B*). The animals were killed on day 31, and the tumors were removed. Fig. 3*C* shows that the average tumor weight in the pANG-RNAi mice was 96 ± 51 mg, representing an 82% reduction from that in the pBS/U6 mice (518 ± 52 mg) (Fig. 3*C*). These results demonstrate that down-

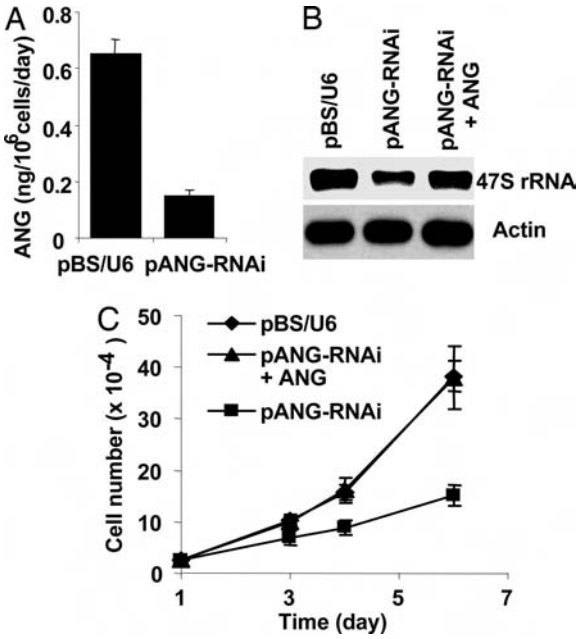

Fig. 2. Down-regulation of angiogenin in PC-3 cells inhibits rRNA transcription and cell proliferation. PC-3 cells were transfected with an angiogenin RNAi plasmid, pANG-RNAi, or with the vector control pBS/U6. Stable transfectants were selected with 0.5 μg/ml puromycin for 2 weeks. (*A*) Secreted angiogenin levels determined by ELISA. (*B*) The steady-state level of 47S rRNA determined by Northern blotting with actin mRNA as the loading control. (*C*) Cell proliferation as determined with a Coulter counter. When present, exogenous angiogenin was 1 μg/ml.

regulation of angiogenin expression decreases the tumorigenicity of PC-3 cells.

IHC staining with mAb 26-2F shows that tumor tissue derived from the pANG-RNAi transfectants (Fig. 4*B*) has a significantly lower angiogenin protein level than that from the vector control transfectants (Fig. 4*A*). Nuclear accumulation of angiogenin is more obvious in the control tumors than in the RNAi transfectant tumors (Fig. 4 *A* and *B*). IHC staining with an anti-PCNA (proliferating cell nuclear antigen) antibody (Fig. 4 *C* and *D*) showed that the percentage of PCNA-positive cells decreased from 74 ± 5 to 37 ± 13 after angiogenin RNAi transfection. Neovessel densities in the vector control and RNAi transfected tumors, as shown by anti-VWF (von Willebrand factor) staining (Fig. 4 *E* and *F*), are 36 ± 3 and 15 ± 2 vessels per mm^2, respectively. These results indicate that both cell proliferation and tumor angiogenesis are decreased in the tumors derived from the pANG- RNAi transfectants.

Prevention of Nuclear Translocation of Angiogenin Inhibits PC-3 Xenograft Tumor Growth in Mice. We have shown that neomycin, an aminoglycoside antibiotic, prevents nuclear translocation of angiogenin in endothelial cells and inhibits its mitogenic and angiogenic activity (31). To determine whether blocking nuclear translocation of angiogenin will inhibit PC-3 cell tumor growth, we used an ectopic model to test the effect of neomycin on the growth of PC-3 cells in athymic mice. First, we confirmed that nuclear translocation of angiogenin in PC-3 cells is indeed blocked by neomycin (Fig. 5 *A* and *B*). Fig. 5*C* shows that treatment with neomycin s.c. at a dose of 60 mg/kg of body weight significantly delayed the establishment of PC-3 cell tumors in nude mice. By day 20, all of the animals in the control group had developed palpable tumors, whereas 7 of the 12 mice in the neomycin-treated group were still tumor-free. At the end of the experiment (56 days), half of the mice in the neomycin-treated group remained tumor-free. In those animals that did

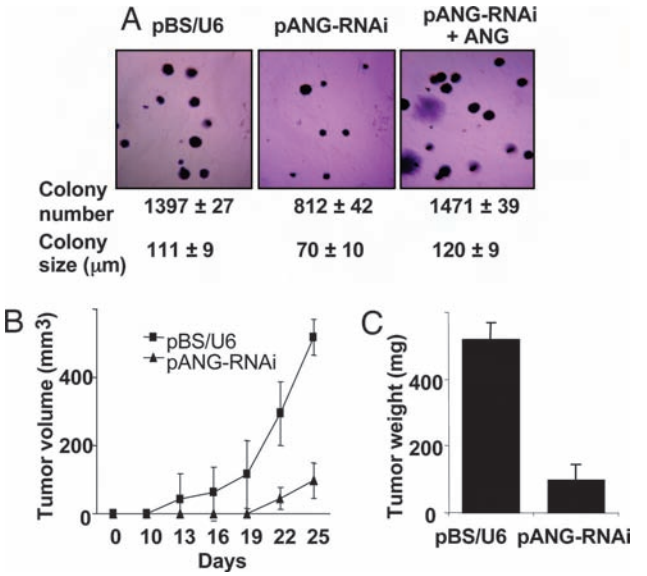

Fig. 3. Knocking down angiogenin expression in PC-3 cells decreases tumorigenicity. (*A*) Soft agar assay in which cells were seeded at a density of 4×10^3 cells per 35-mm dish and cultured in 0.35% soft agar in DMEM plus 10% FBS at 37°C for 7 days. When present, angiogenin was added to both the soft agar and the medium at 0.1 μg/ml. The colonies were stained with 0.05% crystal violet. Colony numbers in the entire dish were counted. The average colony size was determined by measuring the diameters of colonies in 10 microscope fields with a microcaliper. (*B* and *C*) Xenograft growth of PC-3 tumors in nude mice. The vector control (pBS/U6) and the angiogenin RNAi (pANG-RNAi) transfectants (1×10^6 cells per mouse) were injected s.c. (eight mice per group) into 6-week-old male athymic mice. (*B*) Mice were checked daily for tumor appearance by palpation, and tumor volume was measured every 3 days. (*C*) Tumors were removed on day 31 and weighed.

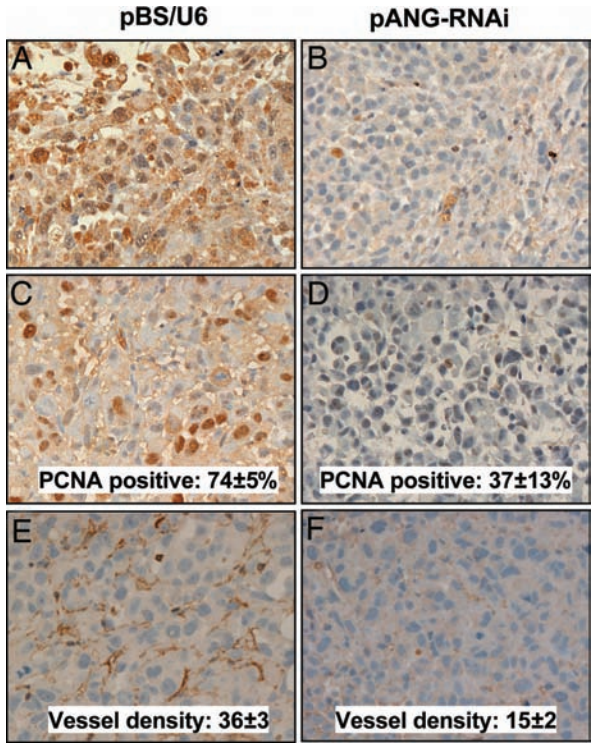

Fig. 4. IHC staining of angiogenin, PCNA, and neovessels. Thin sections (4 μm) from formalin-fixed, paraffin-embedded tumor tissues derived from vector-transfected PC-3 cells (*A*, *C*, and *E*) and from angiogenin RNAi-transfected PC-3 cells (*B*, *D*, and *F*) were stained with antiangiogenin (*A* and *B*), anti-PCNA (*C* and *D*), and anti-VWF (*E* and *F*) antibodies. The bound primary antibodies were visualized with Dako's Envision system. VWF-positive vessels in each tumor were counted in the five most vascularized areas at ×200 magnification, and the numbers were averaged. Vessel density (vessels per field) is shown as mean ± SD for each group. PCNA-positive and total numbers of cells were counted in five randomly selected areas at ×200 magnification. Images shown were from a representative animal of each group.

develop tumors, the average tumor weight was 58 ± 34 mg, which is 23% of that of the control group (253 ± 56 mg) (Fig. 5*C*). PCNA-positive cells in the tumor tissues from control and neomycin-treated animals were 75 ± 5% and 30 ± 6%, respectively, indicating that proliferation of PC-3 cells was inhibited by neomycin (Fig. 5 *E* and *F*). There was a concomitant decrease in tumor angiogenesis after neomycin treatment, as indicated by the neovessel densities determined by IHC staining with an anti-VWF antibody (Fig. 5 *G* and *H*). Neomycin treatment decreased the vessel density from 91 ± 4 to 26 ± 11, representing an 81% inhibition (the relatively high neovessel density in the control was due to the use of Matrigel in these experiments). These results demonstrated that blocking nuclear translocation of angiogenin effectively inhibits PC-3 cell tumor growth in mice, presumably through inhibition of both tumor cell proliferation and angiogenesis.

Discussion

The results presented here indicate that angiogenin plays a dual role in prostate cancer. In addition to a function in tumor angiogenesis, several lines of evidence show that angiogenin also mediates prostate cancer cell proliferation directly. Knocking down angiogenin expression in PC-3 cells inhibited cell proliferation by 65% (Fig. 2*C*). It is of note that the RNAi construct used in these experiments decreased angiogenin expression only by 82% (Fig. 2*A*). We have tested a number of RNAi sequences targeting different regions of angiogenin mRNA and found that, among stably transfected cells, an ≈80% reduction in angiogenin production was the maximum that could be obtained. Two of the five RNAi sequences failed to produce any transfectants because none of the cells survived. The reasons are not clear at present. One possibility is that angiogenin-mediated rRNA transcription is essential, so cells do not survive when angiogenin is inhibited beyond a certain degree. We have previously shown that inhibition of angiogenin expression beyond a certain level in endothelial cells resulted in cell death (30). We are currently generating prostate-specific angiogenin knockout/knockdown mice to study in detail the function of angiogenin in prostate development and in prostate cancer.

Angiogenin RNAi transfectants have reduced capacities to form colonies and to grow in soft agar (Fig. 3*A*). Thus, both anchorage-dependent and anchorage-independent growth of PC-3 cells was inhibited by down-regulating angiogenin expression. This inhibition can be alleviated completely by exogenous angiogenin (Figs. 2 *B* and *C* and 3*A*). These results indicate that the decreases in cell proliferation and colony formation result from decreased angiogenin expression rather than from a nonspecific action of RNAi. In addition, these results imply that exogenous angiogenin can functionally replace endogenous angiogenin in PC-3 cells. Angiogenin has a signal peptide, so the majority of the protein is secreted (32). To determine whether secretion is a necessary step for angiogenin to mediate cell proliferation, we examined the effect of 26-2F, a neutralizing mAb of angiogenin, on PC-3 cell proliferation. Addition of 26-2F to the culture medium inhibited PC-3 cell proliferation in a dose-dependent manner, whereas a subtype-matched nonimmune IgG had no effect (data not shown). These results suggest that an autocrine action of angiogenin accounts for at least part of the activity of angiogenin in mediating PC-3 cell proliferation.

The effect of angiogenin on PC-3 cell proliferation was confirmed by the *in vivo* results obtained in the xenograft tumor model (Fig. 3 *B* and *C*). The appearance of PC-3 cell tumors from

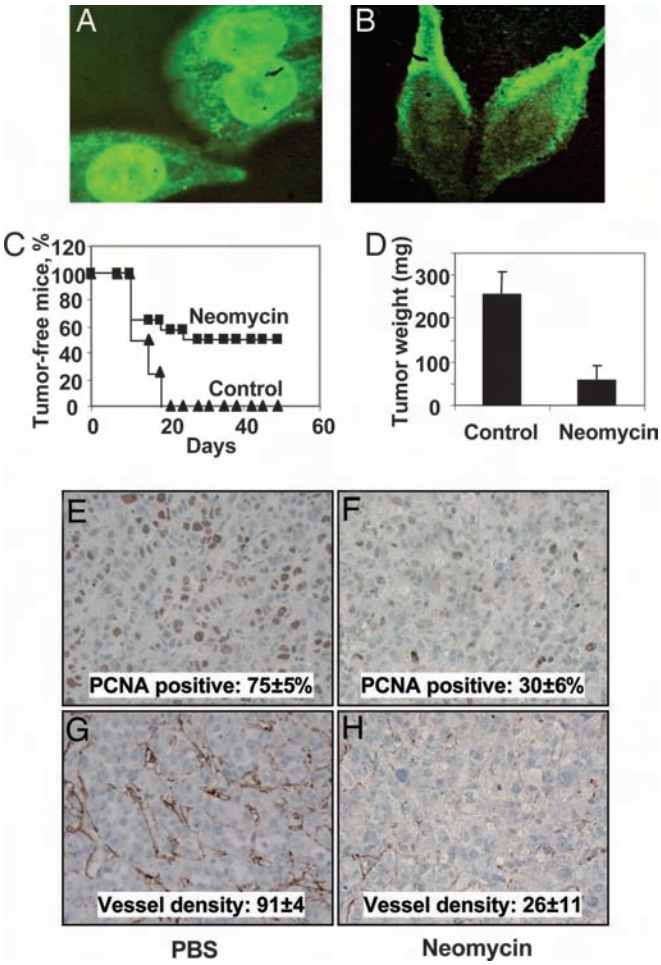

Fig. 5. Effect of neomycin on PC-3 cell tumor growth in athymic mice. (*A* and *B*) Inhibition of nuclear translocation of angiogenin. PC-3 cells were cultured in DMEM plus 10% FBS for 24 h and then incubated with 1 μg/ml angiogenin in the absence (*A*) or presence (*B*) of 100 μM neomycin at 37°C for 30 min. Angiogenin was visualized with 26-2F and Alexa Fluor 488-labeled goat anti-mouse IgG. (*C–H*) Inhibition of tumor growth. PC-3 cells (5×10^5 in 67 μl of Hanks' balanced salt solution) were mixed with 33 μl of Matrigel. The mixture was injected into the left shoulder of the mice. The mice then received s.c. injections of neomycin at a dose of 60 mg/kg of body weight or PBS daily for 2 weeks, followed by injections every other day for another 6 weeks. Twelve mice were used per group. (*C*) Mice were examined daily by palpation for tumor appearance. (*D*) At day 56, mice were killed, and tumor tissues were removed and weighed. (*E* and *F*) Tissue specimens were fixed in 10% formalin, and 4-μm paraffin sections were cut. Proliferating cells were stained with an anti-PCNA mAb. PCNA-positive cells and total numbers of cells were counted in five randomly selected areas at ×200 magnification. (*G* and *H*) Neovessels were stained with an anti-VWF antibody, and neovessels in each tumor were counted in the five most vascularized areas at ×200 magnification.

angiogenin RNAi transfectants was delayed compared with that from the vector control transfectants. IHC staining for PCNA and VWF demonstrated that both cancer cell proliferation and tumor angiogenesis were substantially decreased in the tumors derived from the pANG-RNAi transfectants (Fig. 4). The role of angiogenin in angiogenesis induced by PC-3 cells in xenograft tumors was reported previously. Olson *et al.* (23, 33) showed that an anti-human angiogenin mAb and human angiogenin-specific antisense oligonucleotide inhibited the growth of PC-3 cells injected into the prostate of athymic mice. Based on the fact that angiogenin is a tumor angiogenic protein, the researchers concluded that the observed antitumor effects were due to inhibition of angiogenin-induced tumor angiogenesis. However, our results now suggest that inhibition of cancer cell proliferation may also contribute to the anticancer activity of angiogenin antagonists. We have repeated the experiments with the same antisense oligonucleotide (JF-2S) reported in these studies and found that it actually significantly inhibited PC-3 cell proliferation *in vitro* (data not shown).

IHC staining with 26-2F confirmed that the level of human angiogenin protein is decreased in pANG-RNAi tumors (Fig. 4 *A* and *B*). Consistent with our results with human prostate cancer tissues (Fig. 1), angiogenin was detected in the nucleus of the cancer cells derived from pBS/U6 vector control transfectants. Angiogenin staining is dramatically enhanced in prostate cancer tissues as compared with that in normal prostate tissues and in BPH. The other striking difference in the staining pattern of angiogenin is that there is strong nuclear staining in prostate cancer tissues. Tables 1 and 2 show that, among the various cancer types that we have surveyed, prostate and cervical cancers are the two types where nuclear accumulation of angiogenin is increased most significantly compared with normal tissue. Nuclear angiogenin is prominent in glioma and in malignant peripheral nerve sheath tumors. However, the normal cerebral cortex and normal peripheral nerve contain significant amounts of nuclear angiogenin. We believe our report to be unique in its indication that nuclear angiogenin is detectable in clinical prostate cancer tissue samples. In fact, breast cancer is the only other cancer type that has been reported to contain nuclear angiogenin (27).

The function of nuclear angiogenin is most likely related to rRNA transcription. In endothelial cells, angiogenin undergoes rapid translocation to the nucleus, where it binds to the promoter region of rRNA and stimulates rRNA transcription. An angiogenin-binding element has been identified and has been shown to have angiogenin-dependent promoter activity in a luciferase reporter assay. Consistent with the hypothesis that stimulation of rRNA transcription is one of the mechanisms by which angiogenin mediates prostate cancer growth, pANG-RNAi transfectants have reduced rRNA transcription that can be restored by adding exogenous angiogenin (Fig. 2*B*).

To investigate whether blocking nuclear translocation of angiogenin may have therapeutic value against prostate cancer, we examined the inhibitory activity of neomycin against xenograft growth of PC-3 cells in athymic mice. We have shown that neomycin blocks nuclear translocation of angiogenin, inhibits angiogenin-induced endothelial cell proliferation and angiogenesis (31), and suppresses xenograft growth of some human cancer cells (HT-29, MD-435, and A432) in athymic mice (34). Data presented in Fig. 5 demonstrate that neomycin blocks nuclear translocation of angiogenin in PC-3 cells and inhibits tumor establishment and growth in athymic mice, accompanied by a marked decrease in cancer cell proliferation and tumor angiogenesis.

Prevailing evidence suggests that the function of angiogenin in mediating both endothelial cell and cancer cell proliferation is related to rRNA transcription and depends on nuclear translocation (28, 30). Nuclear translocation of angiogenin in endothelial cells depends strictly on cell density. It decreases as the cell density increases and diminishes when cells are confluent (35). However, nuclear translocation of angiogenin in cancer cells seems to be density-independent (28). We have proposed that one of the reasons for this difference is that cancer cells are able to proliferate independently of cell density and thus require a constant supply of ribosomes. Constitutive nuclear translocation of angiogenin would meet this high metabolic demand of cancer cells. Blocking nuclear translocation of angiogenin in endothelial and cancer cells will inhibit tumor angiogenesis and prostate cancer cell proliferation, respectively, and thus will have a combined benefit of chemotherapy and antiangiogenesis therapy. We are currently evaluating the therapeutic value of neomycin and neamine, the nontoxic derivative of neomycin, against spontaneous prostate cancers in mice.

Materials and Methods

Cells and Transfectants. PC-3 cells were cultured in DMEM plus 10% FBS. The cells were transfected with pANG-RNAi, an angiogenin RNAi plasmid that has been shown to knock down angiogenin expression in HeLa cells (28). This plasmid or the control vector pBS/U6 was cotransfected with pBabe-puro, a plasmid containing the puromycin resistance gene, into PC-3 cells in the presence of Lipofectin. Stable transfectants were selected with 0.5 μg/ml puromycin for 2 weeks. Pooled populations of the vector and the RNAi transfectants were used in this study.

Cell Proliferation Assays. Anchorage-dependent cell proliferation was determined by counting the cell numbers with a counter from Coulter (Hialeah, FL). Anchorage-independent cell proliferation was determined by a soft agar assay. Cells were seeded at a density of 4×10^3 cells per 35-mm cell culture dish in 0.35% agar and cultured for 7 days at 37°C under 5% CO_2. Dishes were stained with 0.05% crystal violet overnight at 4°C. Colonies were counted in the entire dish, and the colony size was determined by a microcaliper. The two-tailed Student *t* test was used to determine the differences between the groups. When exogenous angiogenin was present, it was added when the cells were seeded and was replenished every time the medium was changed.

ELISA Detection of Human Angiogenin. A double-antibody ELISA method (36) was used to measure the angiogenin content in the medium. Cell culture media were collected, and the volumes were normalized to the cell numbers. ELISA plates were coated with 1 μg of antiangiogenin mAb 26-2F per well and blocked with 5 mg/ml BSA in PBS. Samples (100 μl) were added to the wells, and the plates were incubated at 4°C overnight, washed with PBS five times, and incubated with 100 μl of antiangiogenin polyclonal antibody R112 per well (1:4,000) at room temperature for 2 h. The plate was washed four times with PBS and incubated with an alkaline phosphatase-labeled goat anti-rabbit antibody (1.25 μg/ml) at room temperature for 1 h. After washing four times with PBS, 100 μl of 5 mg/ml *p*-nitrophenyl phosphate in 0.1 M diethanolamine containing 10 mM $MgCl_2$ (pH 9.8) was added, and the absorbance at 410 nm was measured. A standard curve of recombinant human angiogenin at concentrations ranging from 50 to 1,000 pg per well was performed each time on every plate.

Xenograft Growth of PC-3 Cell Tumors in Athymic Mice. Outbred male athymic mice (*nu/nu*) were obtained from Charles River Laboratories (Wilmington, MA). The vector control and the RNAi transfectants (1×10^6 cells per mouse) were injected s.c. in the right shoulder. Eight mice per group were used. Tumor sizes were measured every 3 days and recorded in mm^3 (length $\times$ width^2). Mice were killed on day 31, and the wet weight of the PC-3 tumor was recorded. For tumor therapy experiments, the mice were inoculated s.c. with 100 μl of a mixture containing 5×10^5 PC-3 cells and 33 μl of Matrigel. The mice were treated s.c. with neomycin (60 mg/kg) or PBS daily for 2 weeks and then every other day until day 56, at which time the animals were killed and the tumors were removed. Twelve mice were used in each group.

IHC. Microarray slides of human prostate cancer and BPH tissues, human multitumor tissues, and human normal tissues were from Zymed Laboratories (South San Francisco, CA). PC-3 cell tumors were fixed in 10% formalin and embedded in paraffin, and thin sections (4 μm) were cut. The tissue microarray slides and the thin sections of PC-3 cell tumor were deparaffinized with xylene, rehydrated in ethanol, and microwaved for 15 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase was blocked by treatment with 0.3% H_2O_2 in methanol for 30 min. The slides were blocked in 5% dry milk for 10 min, incubated with the primary antibodies at 4°C overnight, and visualized with the Envision system (Dako, Carpinteria, CA). The slides were counterstained with hematoxylin. Negative controls were obtained by omission of the primary antibodies. Angiogenin staining was performed with 26-2F as the primary antibody at a concentration of 30 μg/ml. Proliferating cells were stained with an anti-PCNA mAb (PC10, Dako) at 1:200 dilution. The number of PCNA-positive cells and the total number of cells were counted in five randomly selected areas at $\times$200 magnification. Neovessels were stained with a polyclonal anti-human VWF antibody (Dako) at 1:200 dilution. VWF-positive vessels in each tumor were counted in the five most vascularized areas at $\times$200 magnification (i.e., with a $\times$20 objective lens and a $\times$10 ocular lens; 0.785 mm^2 per field).

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